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Enclosed is a copy of Priority Document 97202303.0 EP filed July 23, 1997 for the above referenced application.

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

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Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécificée à la page suivante.

Patentanmeldung Nr. **Patent application No.** **Demande de brevet n°**

97202303.0 / EP97202303

The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP97202303

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
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If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

The HA-1 antigen

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Title: The HA-1 antigen

The invention relates to the field of immunology, in particular to the field of cellular immunology.

Bone marrow transplantation (BMT), one of the areas the invention is concerned with and the area from which the present invention originates, finds its application in the treatment of for instance severe aplastic anaemia, leukaemia and immune deficiency diseases.

In the early days of this technique many transplants failed through rejection of the graft by the host. Transplants that did succeed, however often led to an immune response by lymphocytes present in the graft against various tissues of the host (Graft versus Host Disease (GvHD)).It is now known that the GvHD response is mainly due to the presence of major histocompatibility (H) antigens which present a transplantation barrier. Therefore it is now routine practice to graft only HLA-matched materials (either from siblings or unrelated individuals) resulting in a much improved rate of success in bone marrow transplantation. However, despite this improvement, as well as improvements in pre-transplantation chemotherapy or radiotherapy and the availability of potent immuno-suppressive drugs, about 20-70% of the treated patients still suffer from GvHD (the percentage is age and bone marrow donor dependent). To avoid GvHD it has been suggested to remove the cells (mature T cells) causing said reaction from the graft. This however often leads to graft failure or to recurrence of the original disease. The cells responsible for GvHD are also the cells which often react against the original aberrant cells in for instance leukaemia (Graft versus Leukaemia response).

Since BMT is nowadays mainly carried out with HLA matched grafts, the GvHD which still occurs must be caused by another group of antigens. It is very likely that the group of so called minor H antigens (mHag), which are non-MHC

encoded histocompatibility antigens (unlike the major H antigens) are at least partially responsible for the remaining incidence of GvHD. mHag's have originally been discovered in congeneic strains of mice in tumor rejection and skin rejection studies. In mice, the use of inbred strains has shown that mHag are encoded by almost 50 different allelically polymorphic loci scattered throughout the genome. In humans, although cumbersome to identify, mHag have been shown to exist, but their overall number and complexity remains uncertain. Minor H antigens are most likely quite different from each other and quite different from major H antigens, they are probably a diverse and elusive group of fragments of molecules which are participating in various cellular housekeeping functions. Their antigenicity may come very incidentally, as naturally processed fragments of polymorphic proteins that associate with MHC products. Some of the mH antigens appear to be widely expressed on various tissues throughout the body whereas others show limited tissue distribution.

One of the better known minor histocompatibility antigens is the H-Y antigen. H-Y is an mH antigen that can lead to rejection of HLA-matched male organ and bone marrow grafts by female recipients, and to a higher incidence of GvHD in female-to-male grafts, particularly if the female donor had been previously pregnant. The H-Y antigen may also play a role in spermatogenesis. The human H-Y antigen is an 11 residue peptide derived from SMCY, an evolutionary conserved Y chromosomal protein. Another well known mH antigen that can lead to GvHD is the HA-2 antigen. The human HA-2 antigen is an 9 residue peptide likely derived from a class I myosin. However, the nature of the HA-1 antigen, responsible for a majority of current cases of GvHD has remained elusive sofar. Human bone marrow transplants performed as therapeutical treatment of severe aplastic anaemia, leukaemia and immune deficiency disease became available in the seventies. For the present, the long-term

results of allogeneic bone marrow transplantation (BMT) have greatly improved due to the use of HLA-matched siblings as marrow donors, advanced pretransplant chemoradiotherapy, the use of potent immunsuppressive drugs as Graft-versus-Host-Disease (GVHD) prophylaxis, better antibiotics and isolation procedures. Nonetheless, the results of clinical BMT reveal that the selection of MHC identical donors/recipients is not a guarantee of avoidance of GVHD or disease free survival even when donor and recipient are closely related. Allogeneic BMT especially in adults results, depending on the amount of T cell depletion of the graft, in uptil 80% of the cases in GVHD. In the HLA genotypically identical situation it amounts to 15-35% whereas in the phenotypical HLA matched recipient/donor combinations, the occurrence of GVHD is significantly higher i.e. 50-80%. Disparities for minor Histocompatibility antigens (mHag) between donor and recipient constitute a potential risk for GVHD or graft failure, which necessitate life long pharmacologic immunosuppression of organ and bone marrow transplant recipients. It is also believed that mHag are involved in the "beneficial" side effect of GVHD i.e. the Graft-versus-Leukemia activity. Several reports demonstrated the presence of anti-host mHag specific CTL in patients suffering from GVHD after HLA genotypically identical BMT. In our laboratory, much effort was put into the further characterization of a (small) number of anti-host mHag specific CTLS. Hereto, CTL clones specific for host mHag were isolated from the peripheral blood (PBL) of patients suffering from severe GvHD. mHag HA-1 specific $CD8^+$ CTL clones were originally obtained after restimulation of in vivo primed PBLs from three patients suffering from GvHD after HLA identical but mHag nonidentical BMT. The post BMT CTL lines were cloned by limiting dilution, resulting in the isolation of a large number of mHag-specific CTL clones. Subsequent immunogenetic analyses revealed that the CTL clones (as described above) identified five non-sexlinked

mHag, designated HA-1, -2, -3, -4, -5, which are recognized in a classical MHC restricted fashion. mHag HA-3 is recognized in the presence of HLA-A1 and mHag HA-1, -2, -4 and 5 require the presence of HLA-A2. Segregation studies demonstrated that each of mHag HA-1 to HA-5 is the product of a single gene segregating in a Mendelian fashion and that HA-1 and HA-2 are not coded within the HLA region. The mHag differ from each other in phenotype frequencies: mHag HA-1 appeared relatively frequent (i.e. 69%) whereas mHag HA-2 appeared very frequent (i.e. 95%) in the HLA-A2 positive healthy population. An inventory in five patients of mHag HA-1, -2, -3, -4 and -5 specific anti-host CTL responses after BMT demonstrated in 3 patients clones specific for the mHag HA-1. This observation points towards the immunodominant behaviour of mhag HA-1. With regard to the mHag expressed on different tissues, we observed ubiquitous versus restricted tissue distribution of the mHag analysed. The expression of the mHag HA-1 is restricted to the cells of the haematopoietic cell lineage, such as thymocytes, peripheral blood lymphocytes, B cells, monocytes. Also the bone marrow derived professional antigen presenting cells: the dendritic cells and the epidermal Langerhans calls express the mHag HA-1. The mHag HA-1 is also expressed on clonogenic leukemic precursor cells as well as on freshly isolated myeloid and lymphoid leukemic cells, indicating that mHag specific CTLs are capable of HLA class I restricted antigen specific lysis of leukemic cells. To substantiate the importance of the human mH antigenic systems, we investigated whether the mHag are conserved in evolution between human and non human primates. Hereto, cells from non human primates were transfected with the human HLAA2.1 gene. Subsequent analyses with our human allo HLA-A2.1 and four mhag A2.1 restricted CTL clones revealed the presentation of ape and monkey allo and mHag HY, HA-1 and HA-2 peptides in the context of the transfected human HLA-A2.1 molecule by ape and monkey target cells. This implicates that the HA-1 peptide

is conserved for at least 35 million years. A prospective study was performed in order to document the effect and clinical relevance of mHag in HLA genotypically identical BMT on the occurrence of acute (grade $\geq$ 2) GVHD. The results of the mHag typing using the CTL clones specific for five well defined mHag HA-1 to HA-5 demonstrated a significant correlation between mHag HA-1, -2, -4 and -5 mismatch and GVHD. A significant correlation ($P = 0.024$) with the development of GVHD was observed when analysed for only mHag HA-1. To analyse a putative peptidic nature of the mHag HA-1, we analysed the requirement of the MHC encoded TAP1 and TAP2 gene products for mhag peptide presentation on the cell surface. The transporter genes TAP1 and TAP2 associated with antigen presentation are required for delivery of peptides from the cytosol with the endoplasmic reticulum. The availability of a human celline "T2" lacking both transport and proteasome subunit genes enabled us to study the processing and presentation of human mHag. We demonstrated that the (rat) transport gene products TAP1 and TAP2u were required for processing and presentation of antigenic peptides from the intracellular mH protein HA-1. Information on the TCR repertoire post BMT in man is extremely scarce. We have analysed the composition of the T cell receptor (TCR) V region ofmHag HA-1 specific CD8+ CTL clones by DNA sequencing of the α and β chains. We observed by analyzing TCR usage of 12 clones derived from 3 unrelated individuals that the TcRβ chains all used the TCRβV6S9 gene segment and showed remarkable similarities within the N-D-N regions.

However, until the present invention no one has succeeded in identifying amino acid sequences of antigenic peptides relevant to the mHag HA-1 antigen, nor has anyone succeeded in the identification of the proteins from which this antigen is derived. We have now for the first time identified a peptide which is a relevant part of mHag HA-1.

Thus this invention provides a (poly)peptide comprising a T-cell epitope obtainable from the minor Histocompatibility

antigen HA-1 comprising the sequence VLXDDLLEA or a derivative thereof having similar functional or immunological properties, wherein X represents a histidine (H) or an arginine (R) residue.

The way these sequences are obtained is described in the experimental part. An important part of this novel method of arriving at said sequences is the purification and the choice of the starting material. Said method is therefore also part of the scope of this invention. However, now that the sequence is known, it is of course no longer necessary to follow that method, because the peptides can easily be made synthetically, as is well known in the art. Since routine techniques are available for producing synthetic peptides, it is also within the skill of the art to arrive at analogs or derivatives of the explicitly described peptides, which analogs and/or derivatives may have the same or at least similar functional or immunological properties and or activity. On the other hand analogs which counteract the activity of the explicitly described peptides are also within the skill of the art, given the teaching of the present invention. Therefor derivatives and/or analogs, be it of the same or different length, be it agonist or antagonist, be it peptide-like or peptidomimetic, are part of the scope of this invention.

The invention provides a (poly) peptide which can be functionally presented to the immune system in the context of the HLA-A2.1 molecule. In general peptides presented in such a context vary in length from about 7 to about 15 amino acid residues, and a polypeptide can be enzymatically processed to a peptide of such length. A peptide provided by the invention typically is at least 7 amino acids in length but preferably at least 8 or 9 amino acids. The upper length of a peptide provided by the invention is no more than 15 amino acids, but preferably no more than about 13 or 11 amino acids in length.

A peptide provided by the invention contains the necessary anchoring residues for presentation in the groove of the HLA-A2.1 molecule. An immunogenic polypeptide provided by the invention comprises a 7-15 amino acid long peptide, provided by the invention, optionally flanked by appropriate enzymatic cleavage sites allowing processing of the polypeptide.
A preferred embodiment of the present invention is the peptide with the sequence VLHDDLLEA which induces lysis of the cell presenting it at a very low concentration of peptide present. This does not imply that peptides inducing lysis at higher concentrations are not suitable. This will for a large part depend on the application and on other properties of the peptides, which were not all testable within the scope of the present invention. The peptides and other molecules according to the invention find their utility in that they may be used to induce tolerance of the donor immune system in HA-1 negative donors, so that residual peripheral blood lymphocytes in the eventually transplanted organ or the bone marrow, as it may be does not respond to host HA-1 material in a HA-1 positive recipient. In this way GvHD will be prevented or mitigated. On the other hand tolerance can be induced in HA-1 negative recipients in basically the same way, so that upon receipt of an organ or bone marrow from an HA-1 positive donor no rejection on the basis of the HA-1 material occurs. For tolerance induction very small doses can be given repeatedly, for instance intravenously, but other routes of administration may very well be suitable too. Another possibility is the repeated oral administration of high doses of the peptides. The peptides may be given alone, or in combination with other peptides, or as part of larger molecules, or coupled to carrier materials in any suitable excipients. Further applications of the peptide or derivatives thereof lie in the prophylactic administration of such to transplanted individuals to prevent GvHD. This can be

done with either agonists, possibly in combination with an adjuvant, or with antagonists which may block the responsible cells. This can be done with or without the concomittant administration of TCR derived peptide sequences or of cytokines. Furthermore the peptides according to the invention can be used to prepare therapeutic agents capable of eliminating a subset of cells, directly or indirectly, especially cells of hematopoietic origin. This can be illustrated by the following examples, which refer to leukemia related therapeutic agents.

A HA-1 positive recipient (in bone marrow transplantation) can be subjected to an additional pre-bone marrow transplant conditioning regime. This means that an agent which specifically recognizes a peptide according to the invention (a HA-1 peptide) as presented selectively on hematopoietic cells, which agent induces elimination of the cells presenting said peptide, is administered to the recipient before transplantation. This agent will eliminate all residual cells (leukemic cells) of hematopoietic origin. Such agents include but are not limited to T cells (which are tailor made *ex vivo* by pulsing with the peptides provided by the invention, and optionally provided with a suicide gene) and/or antibodies coupled to toxic moieties.

A HA-1 negative donor for bone marrow transplantation can be vaccinated with a peptide according to the invention, a HA-1 peptide. Upon transplantation to a HA-1 positive recipient, the donor's immune system can eliminate any residual or recurrent HA-1 peptide presenting cells in the recipient which are of course leukemic. This is another example of tailor-made adoptive immunotherapy provided by the invention.

A transplanted HA-1 positive recipient, transplanted with HA-1 negative (or for that matter HA-1 positive) bone marrow and suffering from recurrent disease (relapse), i.e.

HA-1 positive leukemic cells, can be treated with (again) an agent (as above) which specifically recognizes a peptide according to the invention (a HA-1 peptide) as presented on hematopoietic cells, which agent induces elimination of the cells presenting said peptide. In case of HA-1 positive bone marrow being transplanted to the HA-1 positive recipient, it is still essential (in case of recurrent disease) to eliminate all HA-1 positive cells even though this includes the transplanted material, because otherwise the HA-1 positive leukemia will kill the recipient. To avoid the latter case the patient can be re-transplanted, if necessary.

In such therapy protocols it is possible to first employ adoptive immunotherapy with agents (cells, antibodies, etc.) which specifically recognize and eliminate specific peptide expressing cells (e.g leukemic cells) that need to be destroyed, after which in a second phase the patient is reconstituted with BMT cells replacing the killed cells. The invetion thus provides additional (or even substituting) protocols to other therapeutic measures such as radiation.

Other therapeutical applications of the peptide include the induction of tolerance to HA-1 proteins in HA-1 related (auto)immune diseases. On the other hand they may be used in vaccines in HA-1 related (auto)immune diseases.

Diagnostic applications are clearly within the skill of the art. They include, but are not limited to HA-1 typing, detection of genetic aberrances and the like. Specific gene sequences can be detected with various methods known in the art, such as hybridization or amplification with PCR and the like. Immunological detection of peptides has also widely been practiced.

On the basis of the peptide described herein genetic probes or primers can be produced which can be used to screen for the gene encoding the protein. On the other hand such probes will be useful in detection kits as well. On the basis

of the peptide described herein anti-idiotypic B cells and/ or T cells and antibodies are produced. Various techniques, to allow detection of suitable donors or recipients, may be used, based on amplification of HA-1 related nucleic acid sequences or on the immunological detection of HA-1 related peptide sequences. Suitable amplification or detection techniques are known in the art, and the invention enables the production of diagnostic test kits for HA-1 allelic detection and typing. The GvHD associated mH antigen HA-1 is a peptide derived from one protein allele of a di-allelic genetic system. The identification of this mH antigen HA-1 enables prospective HA-1 typing of BMT donors and recipients to improve donor selection and thereby prevention of GVHD induction. All these embodiments have been made possible by the present disclosure and therefor are part of the present invention. The techniques to produce these embodiments are all within the skill of the art.

Furthermore, the identification of the HA-1 antigen allows the production of synthetic HA-1 peptides and peptides functionaly and/or immunologically related thereto. Such peptides (which can include left or right turning residues) are designed and/or generated by various methods known in the art such as peptide synthesis and replacement mapping, followed by functional binding studies. Altered peptide ligands (APL) for the HLA-A2.1 restricted HA-1 epitope enable modification of the HA-1 directed T cell responses and thus modulate and/or mitigate the GvHD associated T cell response. In general, T cells are activated by the interaction of the T cell receptor (TCR) with the antigenic peptide in the context of a MHC molecule and can react with a number of different effector functions. APL can interact with the TCR and change the effector functions of the T cell qualitatively and/or quantitatively. APL, used *in vitro* as well as *in vivo* can act as antagonist or agonist for the TCR and can anergize the T cells specific for the wild type peptide. The HA-1 peptide is used to induce tolerance in the living bone marrow or

organ (kidney, liver, gut, skin, etc.) of HA-1 negative donors for HA-1 positive patients,. In bone marrow transplantation, the peptide (given alone or in combination with others) is used to induce tolerance in the living bone marrow donor. The peptide(s) may be given orally, intravenously, intra-occularly, intranasally or otherwise. In all forms of organ, tissue and bone marrow transplantation, the HA-1 peptide is used to induce tolerance in HA-1 negative recipients.

The invention also provides an analog of the peptide according to the invention which is an antagonist for the activity of T cells recognizing the peptide. Such an analog is obtained using methods and tests known in the art. Furthermore, the invention provides a method for the generation of antibodies, T cell receptors, anti-idiotypic B-cells or T-cells, comprising the step of immunization of a mammal with a peptide or a polypeptide according to the invention, and the antibodies, T-cell receptors, B-cells or T-cells obtainable by the method. Dose ranges of peptides and antibodies and/or other molecules according to the invention to be used in the therapeutical applications as described herein before are designed on the basis of rising dose studies in the clinic in clinical trials for which rigorous protocol requirements exist.

An important advantage of using mHag-specific CTLs in adoptive immunotherapy of for example leukemia lies in their restricted and specific target cell damage. We take advantage of three of the known characteristics of human mHag i.e. 1) MHC-restricted recognition by T cells; 2) variable phenotype frequencies, i.e. mHag polymorphism; and 3) restricted tissue distribution, allowing specific and distinct targeting of mHag HA-1 related therapy. Restrictive HA-1 tissue expression significantly increases the success of adoptive immunotherapy towards various types of cancer, such as small cell lung carcinoma cells which express also the HA-

1 antigen. Moreover, since mHag are clearly expressed on circulating leukemic calls and clonogenic leukemic precursor cells of both myeloid and lymphoid origin, both types of leukemias can be targeted. mHag peptide CTLs can be generated *ex vivo* from mHag-negative BM donors for mHag-positive patients. Peptide-specific CTL clones from an HLA-A1-positive mHag-negative healthy blood donor are generated by pulsing autologous APCs with mHag HA-1 related synthetic peptide. Proliferating clones are expanded and tested for specific cytotoxic activity. Upon transfusion (either pre-BMT as part of the conditioning regimen or post-BMT as adjuvant therapy), the mHag peptide-specific CTLs will eliminate the mHag-positive patient's leukemic cells and, if of the patient's origin, also the patient's hematopoietic cells but will spare the patient's non-hematopoietic cells. If necessary, subsequent mHag-negative donor BMT will restore the patient's hematopoietic system. A universal approach is to generate "prefab" mHag peptide-specific CTLs by using mHag-negative healthy blood donors with frequent HLA-homozygous haplotypes. Patients who are mHag-positive (and their BM donors mHag-negative) and who match the HLA typing of the CTL donor can be treated with these "ready to be used" allo-peptide specific CTLS. Transduction of these CTLs with a suicide gene allows elimination of the CTLs in case adverse effects occur. For the sake of illustration a number of methods and applications is also given below in the experimental part.

Experimental part.

Graft-versus-Host Disease (GvHD) is a frequent and life-threatening complication after allogeneic HLA-identical bone marrow transplantation (BMT). Recipients of HLA-identical bone marrow develop acute or chronic Graft-versus-Host-Disease in respectively 36% and 49% [1,2]. Disparities in genes other than the MHC, referred to as minor histocompatibility (mH) antigens, are clearly involved in the development of

GvHD after HLA-identical BMT. A recent retrospective analysis revealed the significant association between mismatching for the mH antigen HA-1 and the induction of GvHD after HLA-identical BMT [3]. Minor histocompatibility antigens are recognized by MHC restricted T cells and were shown to be peptides derived from intracellular proteins presented by MHC molecules [4-6]. Here we report the first identification of a polymorphic gene encoding an human mH antigen. The GvHD associated mH antigen HA-1 is a nonapeptide derived from the di-allelic KIAA0223 gene. The HA-1 allelic counterpart encoded by the KIAA0223 gene differs only at one amino acid from the mH antigen HA-1. Family studies demonstrated an exact correlation between the KIAA0223 gene polymorphism and the HA-1 phenotype as was previously determined by recognition by the HA-1 specific CTL clones. The elucidation of the HA-1 encoding gene enables prospective HA-1 DNA typing of BMT donors and recipients to improve donor selection and prevention of GvHD.

Cytotoxic T cell clones specific for the mH antigen HA-1 have been isolated from three different patients with severe GvHD [7]. The mH antigen HA-1 is presented in the context of HLA-A2.1 and present in 69% of the HLA-A2.1 positive population [7]. HA-1 expression was demonstrated to be tissue specific and limited to cells of haematopoietic origin, including dendritic cells, Langerhans cells and leukemic cells [8-10]. Family analysis indicated a mendelian mode of inheritance for HA-1 and segregation independent from the MHC complex [11]. Comparison of the T cell receptor (TCR) sequences of different HA-1 specific T cell clones derived from different individuals revealed conserved usage of the TCR Vβ6.9 and conserved amino acids in the CDR3 region [12]. In a retrospective study, mismatching for a number of mH antigens was evaluated with regard to the association with GvHD after HLA-identical BMT. A single HA-1 mismatch between donor and recipient was significantly correlated with the induction of GvHD after HLA-identical BMT [3].

To identify the mH antigen HA-1, HLA-A2.1 molecules were purified from two HA-1 expressing EBV-transformed B lymphoblastoid cell lines (EBV-BLCL) Rp and Blk. The HLA-A2.1 bound peptides were isolated by acid treatment and fractionation of the peptides was performed by multiple rounds of reverse phase HPLC. The fractions were analysed for their capacity of inducing HA-1 specific lysis using T2 cells as target cells and an HA-1 specific CTL clone as effector cells in a ^{51}Cr-release assay (Fig 1a). Fraction 24 contained HA-1 activity and was two times further fractionated with reverse phase HPLC using a different organic modifier (Fig 1b.c.). Fraction 33 and 34 of the third HPLC fractionation showed HA-1 activity ^{51}Cr-release assay and were analysed by tandem mass spectrometry. Because over a 100 different peptides were present in these fractions, around 40% of fractions 33 and 34 was chromatographed with an on-line microcapillary column effluent splitter. The fractions were simultaneously analysed by tandem mass spectrometry and ^{51}Cr-release assay (Fig 1d.). Five peptide species (at m/z 550, 520, 513, 585 and 502) were specifically present in active fractions and absent in fractions without activity in the CML assay. Collision activated dissociation analysis of peptide candidate m/z 550 revealed the sequence YXTDRVMTV. X stands for Isoleucine or leucine that cannot be discriminated with this type of mass spectrometer. However, a synthetic peptide with this sequence was not able to reconstitute the HA-1 epitope (results not shown). To determine which of the four remaining candidates was the HA-1 peptide the second HA-1 purification of the EBV-BLCL Blk was evaluated. HA-1 positive peptide fraction 33 of the second reverse phase HPLC fractionation was further chromatographed by microcapillary HPLC with a third organic modifier. A single peak of reconstituting activity was observed in a ^{51}Cr-release assay (results not shown). Mass spectral analysis of these fractions revealed that only peptide candidate m/z 513 was present. This peptide was analyzed with collision activated

dissociation analysis and sequenced as VXHDDXXEA (Fig 2a). Isoleucine and Leucine variants of the peptide were synthesized and run on the microcapillary HPLC column. Only peptide VLHDDLLEA coeluted with the naturally processed peptide 513 (results not shown). Next, synthetic VLHDDLLEA added in different concentration to a CML assay with 3 different HA-1 specific CTL clones revealed recognition by all three clones of the peptide with a half maximal activity at 150-200 pM for or all three clones (Fig 2b). This demonstrated that the mH antigen HA-1 is represented by the nonapeptide VLHDDLLEA.

Database searches performed to identify the gene encoding HA-1, revealed that the HA-1 peptide VLHDLLEA was identical for 8 out of 9 amino acids with the peptide VLRDDLLEA from the KIAA0223 partial complementary DNA (cDNA) sequence, derived from the acute myelogenous leukemia KG-1 cellline. Because HA-1 has a population frequency of 69%, we reasoned that the VLRDDLLEA peptide sequence might represent the HA-1 allelic counterpart present in the remaining 31% of the population. To elaborate on this assumption, we performed cDNA sequence analysis of the putative HA-1 encoding region of KIAA0223 in EBV-BLCL derived from a presumed HA-1 homozygous positive (vR), from a presumed HA-1 negative individual (DH) and from the KG-1 cell line (Table 1.). The HA-1 encoding region of KIAA0223 of the HA-1+/+ individual (vR) displayed two nucleotides differences from the KIAA0223 sequence in the databank, leading to the amino acid sequence VLHDDLLEA (designated HA-1^H). The HA-1 encoding region of KIAA0223 of the HA-1-/- individual (DH) showed 100% homology with the reported KIAA0223 sequence (designated HA-1^R). The KG-1 cell line expressed both KIAA0223 alleles. Because KG-1 does not express the restriction molecule HLA-A2.1 necessary for T cell recognition, we transfected KG-1 with HLA-A2.1 and used these cells as target cells in a ^{51}Cr-release assay with the HA-1 specific T cell clone as effector cells. According to the cDNA sequence analysis results, the KG-1 cells were

recognized by the HA-1 specific T cell clone (data not shown). This result suggested that the KIAA0223 gene forms a di-allelic system of which the HA-1^H allele leads to recognition by the mH antigen HA-1 specific T cell clones.

Two families, who were previously typed for HA-1 with HA-1 specific CTL , were studied on the cDNA level for their KIAA0223 polymorphism. The family members of family 1. were screened for their KIAA0223 sequence polymorphism by sequencing the HA-1 encoding sequence region. All HA-1 negative members displayed the HA-1^R sequence, whereas all HA-1 positive members turned out to be heterozygous, thus carrying both HA-1 alleles (Fig3a). We subsequently designed HA-1 allele specific PCR primers to screen another family previously cellularly typed for HA-1. Both parents and one child were determined as heterozygous for HA-1, two HA-1 negative children homozygous for the HA-1^R allele and one child homozygous for the HA-1^H allele (Fig.3b). The screening of both families showed an exact correlation of the HA-1 phenotype as determined by recognition by the HA-1 specific T cell clones and the KIAA0223 gene polymorphism.

To definitely prove that the KIAA0223 gene encodes the mH antigen HA-1, the HA-1 encoding sequence region of KIAA0223 of both the HA-1^H and the HA-1^R alleles were cloned in a eukaryotic expression vector and transiently transfected in HA-1 negative Hela cells in combination with HLA-A2.1. HA-1 specific T cell recognition of these transfected Hela cells was assayed using a TNFα release assay. The Hela cells transfected with the HA-1^H sequence containing vector were recognized by two HA-1 specific T cell clones (Fig.3c). In contrast transfection of the HA-1^R sequence containing vector did not lead to recognition. In conclusion, our results clearly demonstrate that the mH antigen HA-1 is encoded by the HA-1^H allele of the KIAA023 gene.

Reconstitution and HLA-A2.1 binding assays were performed to determine the capacity of HA-1^R peptide

VLRDDLLEA to bind to HLA-A2.1 and to be recognized by the HA-1 specific T cell clones. The concentration of the HA-1^R peptide that inhibited the binding of a fluorescent standard peptide to HLA-A2.1 by 50 % (IC50) was 365 nM, falling in the intermediate binders, whereas the IC50 of the HA-1^H peptide was 30 nM, which is in the range of high affinity binders (Fig. 4a) [13,14]. Different concentrations of VLRDDLLEA were tested in a ^{51}Cr-release assay with three HA-1 specific T cell clones. One out of three clones (3HA15) tested showed recognition of the HA-1^R peptide, but only at 1000 times higher peptide concentration than that necessary for the recognition of the HA-1^H peptide (Fig 4b). As the binding affinity of the two peptides to HLA-A2.1 differs only 10-fold, it can be concluded that all the T cell clones specifically recognize the HA-1^H peptide.

The 3HA15 T cell clone, recognizing the HA-1^R peptide at high concentrations, does not recognize HA-1^R homozygous individuals. This suggests that the VLRDDLLEA peptide is not presented by HLA-A2.1 or presented below the detection limit of the T cell. To determine whether the HA-1^R peptide VLRDDLLEA was presented by HLA-A2.1, HLA-A2.1 bound peptides were eluted from a HA-1^R homozygous EBV-BLCL and fractionated with reverse phase HPLC. The synthetic HA-1- peptide VLRDDLLEA was run on reverse HPLC to determine at which fraction this peptide eluted. The corresponding HPLC fractions derived from the HA-1^R expressing EBV-BLCL were analysed using mass spectrometry. Presence of peptide VLRDDLLEA could not be detected (results not shown), indicating that this peptide is not or in very low amounts presented by HLA-A2.1 on the cell surface. This is most likely due to the 10-fold lower binding affinity of the peptide for HLA-A2.1. The supposed absence of the HA-1^R peptide in HLA-A2.1 indicate that this allele must be considered as a null allele with regard to T cell reactivity. This implicates that only BMT from an HA-$1^{R/R}$ (HA-1-) donor to HA-$1^{H/H}$ or HA-$1^{R/H}$ (HA-1+) recipient direction and not the

reverse would be significantly associated with GvHD. This is indeed observed in a retrospective study in which HLA-2.1 positive BMT pairs were typed for HA-1 [3]. However, HA-1^R derived peptides may bind to other HLA alleles and possibly be recognized by T cells. If the latter peptides are not generated and presented by the HA-1^H allele, then T cell reactivity towards the HA-1^R allele may be envisaged and GvHD in that direction may occur.

Only a few murine and human mH antigens have been identified so far on the peptide and gene level. Two murine mH antigens are encoded by mitochondral proteins, leading to respectively four and two alleles [15-17]. In addition, two murine H-Y mH antigens were shown to be peptides encoded by Y-chromosome located genes [18-21]. The human *SMCY* gene, located on the Y chromosome, encodes the HLA-B7 and the HLA-A2.1 restricted H-Y mH antigens [5,6]. Of the human non-sex linked mH antigens only the mH antigen HA-2 has been sequenced on the peptide level, but the HA-2 encoding gene remained unknown [4]. The identification of the gene encoding the mH antigen HA-1 is the first demonstration that human mH antigens are derived from polymorphic genes. The HA-1 encoding KIAA0223 gene has two alleles differing in two nucleotides leading to one single amino acid difference. However, because the KIAA0223 gene has not been fully sequenced yet, it remains to be established whether additional amino acid polymorphisms between the two alleles of this gene are present.

Because the HA-1 mH antigen is the only known human mH antigen that is correlated with the development of GvHD after BMT the results of our study are of significant clinical relevance [3]. Although the numbers of different human mH antigens is probably high, it is envisaged that only few immunodominant mH antigens can account for the risk for GvHD [22]. Identification of those human immunodominant mH antigens and screening for those antigens may result in a significant decrease in GvHD after BMT. Here we describe the first elucidation of a polymorphic gene encoding the immunodominant

mH antigen HA-1. This enabled us to design HA-1 allele specific PCR primers for pre-transplant donor and recipient typing to improve donor selection and thereby prevention of HA-1 induced GvHD development.

Methods

Cell culture. The CD8+ HLA-A2.1 restricted HA-1 specific cytotoxic T cell clones 3HA15, clone 15 and 5W38 were derived from PBMC of two patients who had undergone HLA identical bone marrow transplantation [7,23]. The clones were cultured by weekly stimulation with irradiated allogeneic PBMC and BLCL in RPMI-1640 medium containing 15 % human serum, 3 mM l-glutamin, 1% Leucoagglutinin-A and 20 U/ml rIL-2. The HLA-A2.1 positive HA-1 expressing EBV transformed B cell lines (BLCL) Rp and Blk were maintained in IMDM containing 5% FCS. The KG-1 and T2 cell lines were cultured in 1640 medium containing 3 mM l-glutamin and 10% FCS.

^{51}Cr-release assay. HPLC fractions and synthetic peptides were tested in a ^{51}Cr-release assay as described [24]. 2500 ^{51}Cr labeled T2 cells in 25 µl were incubated with 25 µl peptide dissolved in Hanks 50mM Hepes for 30 minutes at 37°C. Cytotoxic T cells were added in an endvolume of 150 µl. When HPLC peptide fractions were tested, T2 was incubated with 2 µg/ml MA2.1 during the ^{51}Cr labelling. After 4 hours at 37°C the supernatants were harvested.

Peptide purification. Peptides were eluted out of purified HLA-A2.1 molecules as earlier described [24]. Briefly, HLA-A2.1 molecules were purified two times from 90.10^9 HLA-A2.1 positive EBV-BLCL by affinity chromatography with BB7.2 coupled CNBR-activated sepharose 4B beads (Pharmacia LKB) and extensively washed. Peptides were eluted from the HLA-A2.1 with treatment with 10% acetic acid, further acidified by 1% TFA and separated from the HLA-A2.1 heavy chain and β2-microglobulin by filtration over a 10kD Centricon (Amicon) filter. Peptides were fractionated using reverse phase micro

HPLC (Smart System, Pharmacia). For the first purification three rounds of HPLC fractionation were used to purify the HLA-A2.1 restricted HA-1 active peptide fractions from 90.10^9 Rp cells. The first fractionation consisted of buffer A: 0.1% HFBA in H2O, buffer B: 0.1% HFBA in acetonitrile. The gradient was 100% buffer A (0 to 20 min), 0 to 15% buffer B (20 to 25 min) and 15 to 70% buffer B (25 to 80 min) at a flow rate of 100 µl/min. Fractions of 100 µl were collected. Fraction 24 of the first gradient was further fractionated. The second fractionation consisted of buffer A: 0.1% TFA in H2O, buffer B: 0.1% TFA in acetonitrile. The gradient was 100% buffer A (0 to 20 min), 0 to 12% buffer B (20 to 25 min), and 12 to 50 % buffer B (25 to 80 min) at a flow rate of 100 µl/min. Fractions of 100 µl were collected. A shallower third gradient was used to further purify fraction 27 that contained HA-1 activity. The gradient was 100% buffer A (0 to 29 min), 0 to 18% buffer B (29 to 34 min), 18% buffer B (34 to 39 min), 18 to 23.9 % buffer B (39 to 98 min) at a flowrate of 100 µl/min. 1/180 to 1/45 of the starting material was used to test for positive fractions in the ^{51}Cr-release assay. Comparable HPLC fractionations were used for the second purification of HLA-A2.1 restricted HA-1 active peptide fractions from 90.10^9 Blk. 40% of the HA-1 containing fraction 33 of the second HA-1 purification was used for an additional reverse phase microcapillary HPLC fractionation. Buffer A was 0.1% triethyl amine (TEA) in water buffered to pH 6.0 with acetic acid and buffer B was 0.085% TEA in 60% acetonitrile buffered to pH 6.0 with acetic acid. The gradient was 100% buffer A (0 to 5 min), 0 to 100% B (5 to 45 min) at a flow rate of 0.5 µl/min. Fractions were collected in 50 µl of 0.1% acetic acid every minute for 5 to 15 minutes, every 30 seconds from 15 to 20 minutes, every 20 seconds from 20 to 40 minutes, and every 30 seconds from 40 to 45 minutes. For each fraction collected, 20% was used to test for HA-1 activity and 80% was used to obtain mass spectral data.

Mass spectrometry. Fractions from third dimension HPLC separation of the Rp purification that contained the HA-1 activity were analyzed by microcapillary HPLC-electrospray ionization mass spectrometry [25]. Peptides were loaded onto a C18 microcapillary column (75µm i.d. x 10 cm) and eluted with a 34 minute gradient of 0 to 60% B, where solvent A was 0.1M acetic acid in water and solvent B was acetonitrile at a flow-rate of 0.5µl/min. One-fifth of the effluent was deposited into the wells of a 96-well plate containing 100 µl of culture media in each well (10 seconds fractions), while the remaining four-fifths was directed into the elctrospray source of the TSQ-70U. Mass spectra and CAD mass spectra were recorded on a Finnigan-MAT TSQ-7000 (San Jose, California) triple quadrupole mass spectrometer equipped with an electrospray ion source.

HLA-A2.1 peptide binding assay. A quantitative assay for HLA-A2.1 binding peptides based on the inhibiton of binding of the fluorescent labeled standard peptide Hbc 18-27 F to C6 (FLPSDCFPSV) to recombinant HLA-A2.1 protein and β2-microglobulin was used [26,27]. In short, HLA-A2.1 concentrations yielding approximately 40-60% bound fluorescent standard peptide were used with 15 pmol/well (150 nM) β2-microglobulin (Sigma). Various doses of the test peptides were coincubated with 100 fmol/well (1 nM) fluorescent standard peptide, HLA-A2.1 and β2-microglobulin for 1 day at room temperature in the dark in a volume of 100 µl in assay buffer. The percent of MHC-bound fluorescence was determined by gel filtration and the 50% inhibitory dose was deduced for each peptide using one-site competition non-linear regression analysis with the prismgraph software. Synthetic peptides were manufactured on a Abimed 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany) and were more than 90% pure as checked by reverse phase HPLC.

RT-PCR amplification and sequencing of KIAA0223 region coding for HA-1

Total or mRNA was prepared from BLCL using the RNAzol methode

(Cinaa/Biotecx Laboratories, Houston, TX) or according to manufacturer's instructions (QuickPrep mRNA purification Kit, Pharmacia Biotech). CDNA was synthesized with 1 µg RNA as template and with KIAA0223 based reverse primer 5'-GCTCCTGCATGACGCTCTGTCTGCA-3'.To amplify the HA-1 region of KIAA0223 the following primers were used: Forward primer 5'-GACGTCGTCGAGGACATCTCCCAT-3' and reverse primer 5'-GAAGGCCACAGCAATCGTCTCCAGG-3'. Cycle parameters used were denaturation 95 °C, 1 min, annealing 58 °C, 1 min and extension 72 °C, 1 min (25 cycles). The PCR-products were purified using the Magic PCR-Preps DNA purification System (Promega) and direct cloned using the pMosBlue T-vector kit (Amersham LIFE SCIENCE). Six independent colonies from each individual were sequenced using the T7-sequencing kit (Pharmacia Biotech) .

HA-1 allele specific PCR amplification

In the case of HA-1 allele specific PCR amplification, cDNA was synthesized as described above. A PCR amplification was performed with allele specific forward primers: for the HA-1^H allele primer H1: 5'-CCT-TGA-GAA-ACT-TAA-GGA-GTG-TGT-GCT-GCA-3', for the HA-1^R allele primer R1: 5'-CCT-TGA-GAA-ACT-TAA-GGA-GTG-TGT-GTT-GCG-3' and for both reaction the reverse primer as described above was used. Cycle parameters used were denaturation 95 °C, 1 min, annealing 67 °C, 1 min and extension 72 °C, 1 min (25 cycles).

Cloning and expression of HA-1^H and HA-1^R allelic region of KIAA0223. A forward KIAA00223 based PCR primer containing an ATG startcodon (5'-CCG-GCA-TGG-ACG-TCG-TCG-AGG-ACA-TCT-CCC-ATC-3') and a reverse KIAA0223 based PCR primer containing a translational stop signal (5'-CTA-CTT-CAG-GCC-ACA-GCA-ATC-GTC-TCC-AGG-3') were designed and used in a RT-PCR reaction with cDNA derived from an homozygous HA-1^H and a homozygous HA-1^R BLCL. Cycle parameters used were denaturation 95 °C, 1 min, annealing 60 °C, 1 min and extension 72 °C, 1 min (25 cycles). The desired PCR-products were purified using the Magic PCR-Preps DNA purification System (Promega). The

purified DNA was direct cloned using the pMosBlue T-vector kit (Amersham LIFE SCIENCE) and recloned in the eukaryotic pCDNA3.1(+) vector under the control of a CMV promotor. Transient cotransfections were performed with HLA-A2.1 in Hela cells using DEAE-Dextran coprecipitation. After 3 days of culture HA-1 specific T cells were added and after 24 hours the TNFα release was measured in the supernatant using WEHI cells [28].

Brief description of the drawings

Table 1. KIAA0223 sequence polymorphism in mH HA-1 positive and HA-1 negative individuals.
Sequencing of HA-1 region in KIAA0223 gene in HA-1 +/+ and HA-1-/- homozygous individuals and KG-1 revealed two alleles differing in two nucleotides resulting in a one amino acid difference (H to R) and designated HA-1^H and HA-1^R . For DH and vR 6 independent PCR products were sequenced. For KG-1 8 PCR products were sequenced.

Figure 1. Reconstitution of HA-1 with HPLC fractionated peptides eluted from HLA-A2.1 molecules in a ^{51}Cr-release assay with mH HA-1 specific T cell clone 3HA15. a. Peptides were eluted from 90.10^9 HA-1 and HLA-A2.1 positive Rp cells and separated using reverse phase HPLC with HFBA as organic modifier. b. Fraction 24 of the first HPLC dimension that contained HA-1 activit
y was further fractionated by reverse phase HPLC with TFA as organic modifier. c. HA-1 containing fraction 27 of the second gradient was further chromatographed with a third shallower gradient consisting of 0.1% acetonitrile/min. Background lysis of T2 by the CTL in the absence of any peptides was in a 3%, in b and c 0%. Positive control lysis was in a 99%, in b 74% and in c 66%. d. Determination of candidate HA-1 peptides. HPLC fraction 33 from the separation in Fig. 1c. was chromatographed with an on-line microcapillary column effluent splitter and analysed by electrospray ionization mass spectrometry and a ^{51}Cr-release assay. HA-1 reconstituting activity as percent specific release was compared with the abundance of peptide candidates measured as ion current.

Figure 2. Sequencing of mH HA-1 peptide by tandem mass spectrometry. a. Collision activation dissociation mass spectrum of peptide candidate with *m/z* of 513. b.

Reconstitution assay with different concentrations of synthetic mH HA-1 peptide with three HA-1 specific T cell clones, 3HA15, clone 15 and 5W38. Background lysis of T2 by the CTL in the absence of any peptide was for 3HA15 4%, for clone 15 10% and for 5W38 2%. Positive control lysis was for 3HA15 46%, for clone 15 47% and 5W38 48%.

Figure 3. KIAA0223 polymorphism exactly correlated with mH antigen HA-1 phenotype. a. The HA-1 region of KIAA0223 was sequenced in a HA-1 mH antigen typed family. 6 PCR products of each family member were sequenced. Family members 00, 07 and 09 expressed the HA-1^R in all 6 PCR products. Family member 01 expressed the HA-1^H allele in 2 PCR products and the HA-1^R allele in 4 PCR products. Family member 02 expressed the HA-1^H allele in 3 PCR products and the HA-1^R allele in 3 PCR products. Family member 08 expressed the HA-1^H allele in 4 PCR products and the HA-1^R allele in 2 PCR products b. HA-1 allele specific PCR reaction in a HA-1 mH antigen typed family correlated exactly with the HA-1 phenotype. The sizes of the resulting PCR products were consistent with the expected sizes deduced from the cDNA sequence. c. Transfection of the HA-1^H allele of KIAA0223 leads to recognition by mH HA-1 specific T cells. The HA-1^H and the HA-1^R coding sequence of KIAA0223 were together with HLA-A2.1 transfected into Hela cells. After 3 days the HA-1 specific CTL clones 5W38 and 3HA15 were added and after the 24 hours TNFα release was measured in the supernatant. The clone Q66.9 is specific for the influenza matrix peptide 58-66. No TNFα production was observed after transfection of the pcDNA3.1(+) vector alone (results not shown).

Figure 4. a. Binding of HA-1^H and HA-1^R peptides to HLA-A2.1. The binding of HA-1^H and HA-1^R peptides were assayed for their ability to inhibit the binding of fluorescent peptide FLPSDCFPSV to recombinant HLA-A2.1 and β2-microglobulin in a cell free peptide binding assay. One representative

experiment is shown. The IC50 is determined on the results of 4 experiments and was 30 nM for VLHDDLLEA and 365 nM for VLRDDLLEA. b. Reconstitution assay with different concentrations of synthetic HA-1^R peptide with HA-1 specific T cells. The HA-1^R peptide was titrated and preincubated with T2 cells. Three HA-1 specific T cell clones, 5W38, 3HA15 and clone 15 were added and a 4 hr ^{51}Cr-release assay was performed. Background lysis of T2 by the CTL in the absence of any peptide was for 3HA15 4%, for clone 15 10% and for 5W38 2%. Positive control lysis was for 3HA15 46%, for clone 15 47% and 5W38 48%.

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Cell	CTL analysis HA-1 phenotype	KIAA0223 sequence	Nr. of clones sequenced	DNA analysis HA-1 phenotype
DH	HA-1-/-	GAGTGTGTGTTGCGTGACGACCTCCTTGAGGCCCGCCG E C V L R D D L L E A R R	(6/6 clones)	HA-1^{R}/HA-1^{R}
vR	HA-1+/+	GAGTGTGTGCTGCATGACGACCTCCTTGAGGCCCGCCG E C V L H D D L L E A R R	(6/6 clones)	HA-1^{H}/HA-1^{H}
KG-1	HA-1+	GAGTGTGTGTTGCGTGACGACCTCCTTGAGGCCCGCCG E C V L R D D L L E A R R	(1/8 clones)	HA-1^{R}/HA-1^{H}
		GAGTGTGTGCTGCATGACGACCTCCTTGAGGCCCGCCG E C V L H D D L L E A R R	(7/8 clones)	

Table 1

CLAIMS

1. A peptide constituting a T-cell epitope obtainable from the minor Histocompatibility antigen HA-1 comprising the sequence VLXDDLLEA or a derivative thereof having similar functional or immunological properties, wherein X represents a histidine or an arginine residue.

2. An immunogenic polypeptide obtainable from the minor Histocompatibility antigen HA-1 comprising the sequence VLXDDLLEA or a derivative thereof having similar functional or immunological properties, wherein X represents a histidine or an arginine residue.

3. A peptide or polypeptide according to claim 1 or 2, comprising the sequence VLHDDLLEA.

4. Vaccine comprising an epitope or a polypeptide according to any one of claims 1-3.

5. A pharmaceutical formulation comprising an epitope or a polypeptide according to any one of claims 1-3.

6. Peptide or polypeptide according to claims 1-3 for use as a medicine.

7. Use of a peptide or polypeptide according to claims 1-3 in the preparation of a medicament for the induction of tolerance for transplants to prevent rejection and/or Graft versus Host disease or to treat (auto)immune disease.

8. A method for the elimination of a group of (neoplastic) hematopoietic cells presenting a peptide in the context of HLA class 1 according to any of one of claims 1-3, whereby elimination is induced directly of indirectly by specific recognition of said peptide in said context.

9. Analog of the peptide according to claim 1, which is an antagonist for the activity of T cells recognizing said peptide.

10. Method for the generation of antibodies, T cell receptors, anti-idiotypic B-cells or T-cells, comprising the step of immunization of a mammal with a peptide or a polypeptide according to claim 1 or 2.

11. Antibodies, T-cell receptors, B-cells or T-cells obtainable by the method of claim 10.

ABSTRACT

The present invention discloses the peptide sequence of a so called minor H antigen. The minor H antigens are associated with the Graft versus Host Disease. The peptide and its derivatives find many uses in bone marrow transplantation, organ transplantation and in the treatment of leukemia. The peptide and its derivatives can be incorporated in vaccines, in pharmaceutical formulations and they can be used in diagnostic test kits. The peptide is derived from the HA-1 minor antigen and has the sequence VLXDDLLEA, wherein X represents a histidine or an arginine residue. Both donors and recipients in bone marrow transplantation can be treated with the peptides, optionally in combination with other peptides, coupled to carriers, with suitable excipients and/or adjuvants.

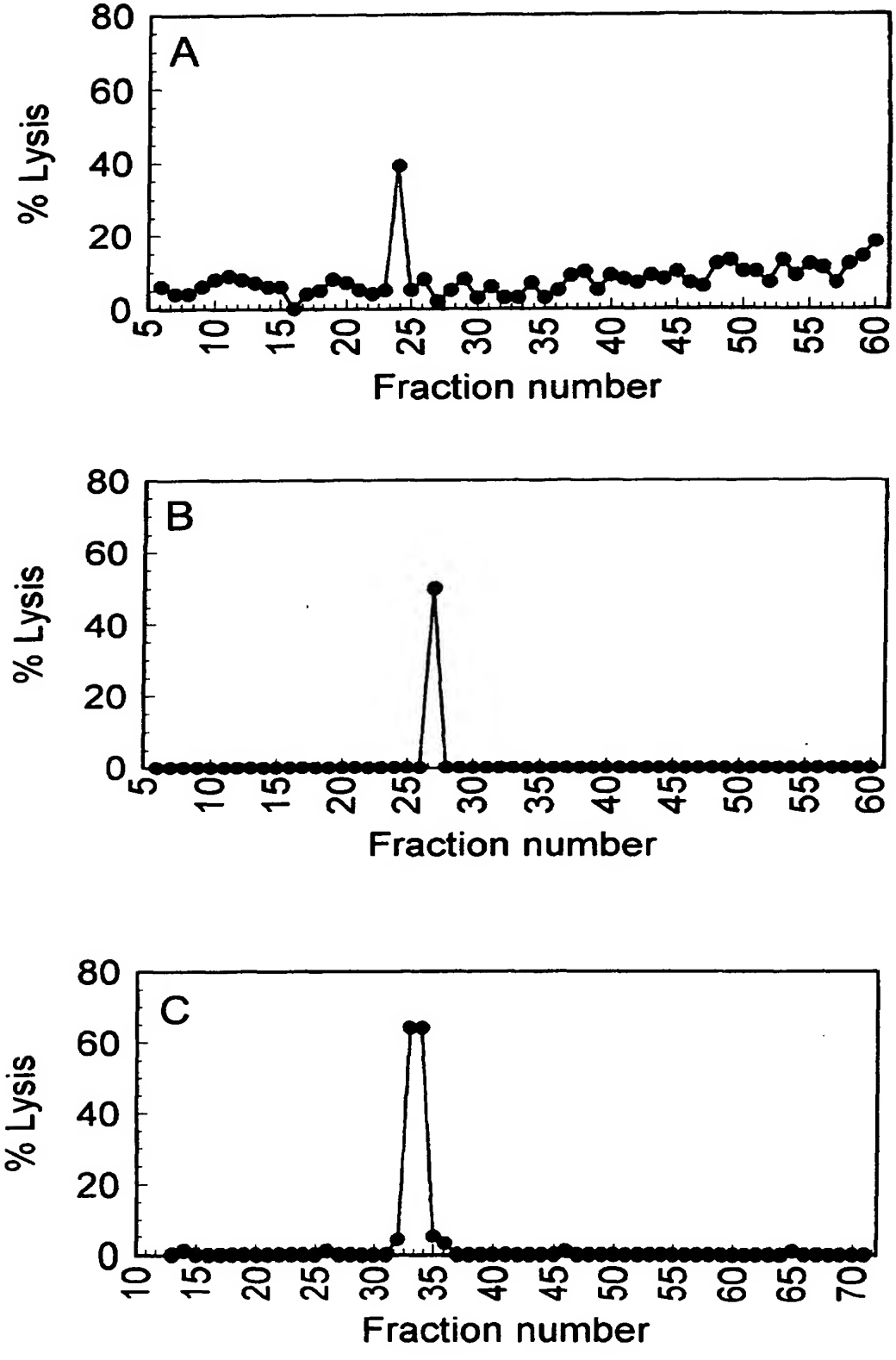
A
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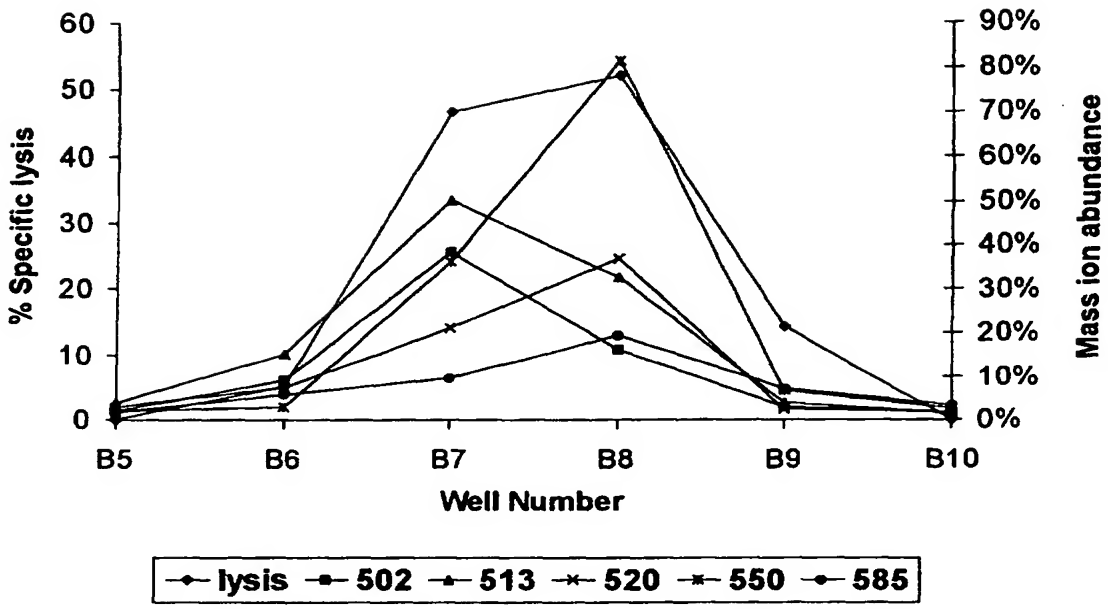
Fig 1


60
50
40
30
20
10
0
% Specific lysis
90%
80%
70%
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50%
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30%
20%
10%
0%
Mass ion abundance
B5
B6
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B8
B9
B10
Well Number
lysis
502
513
520
550
585

Fig 1d

100	213	350	465	580	693	806	935	1024
Val	Xxx	His	Asp	Asp	Xxx	Xxx	Glu	Ala
1024	925	812	675	560	445	332	219	90

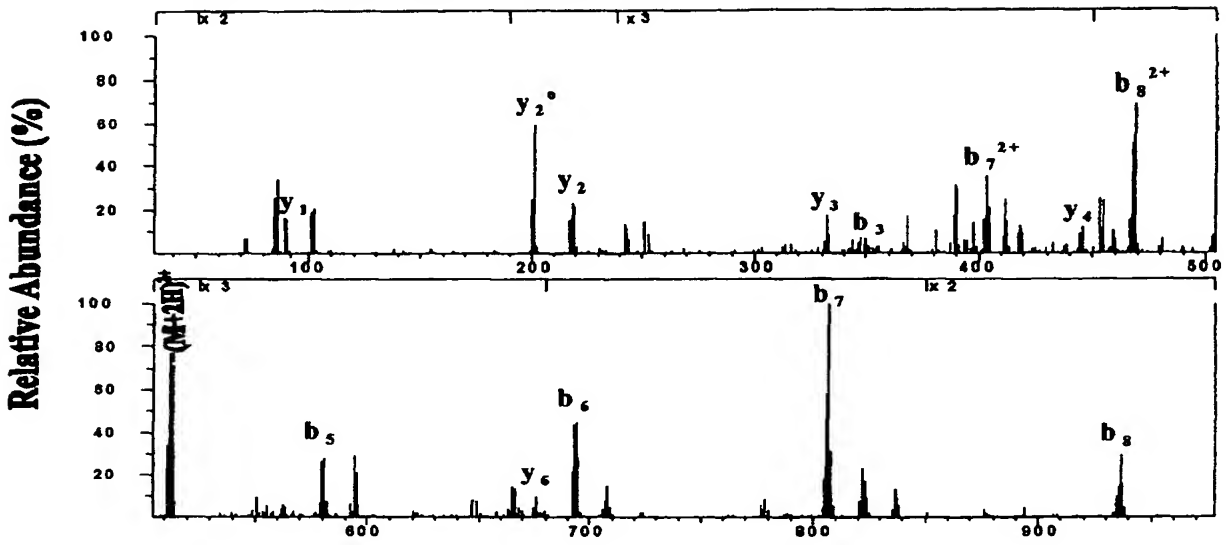


Fig 2A

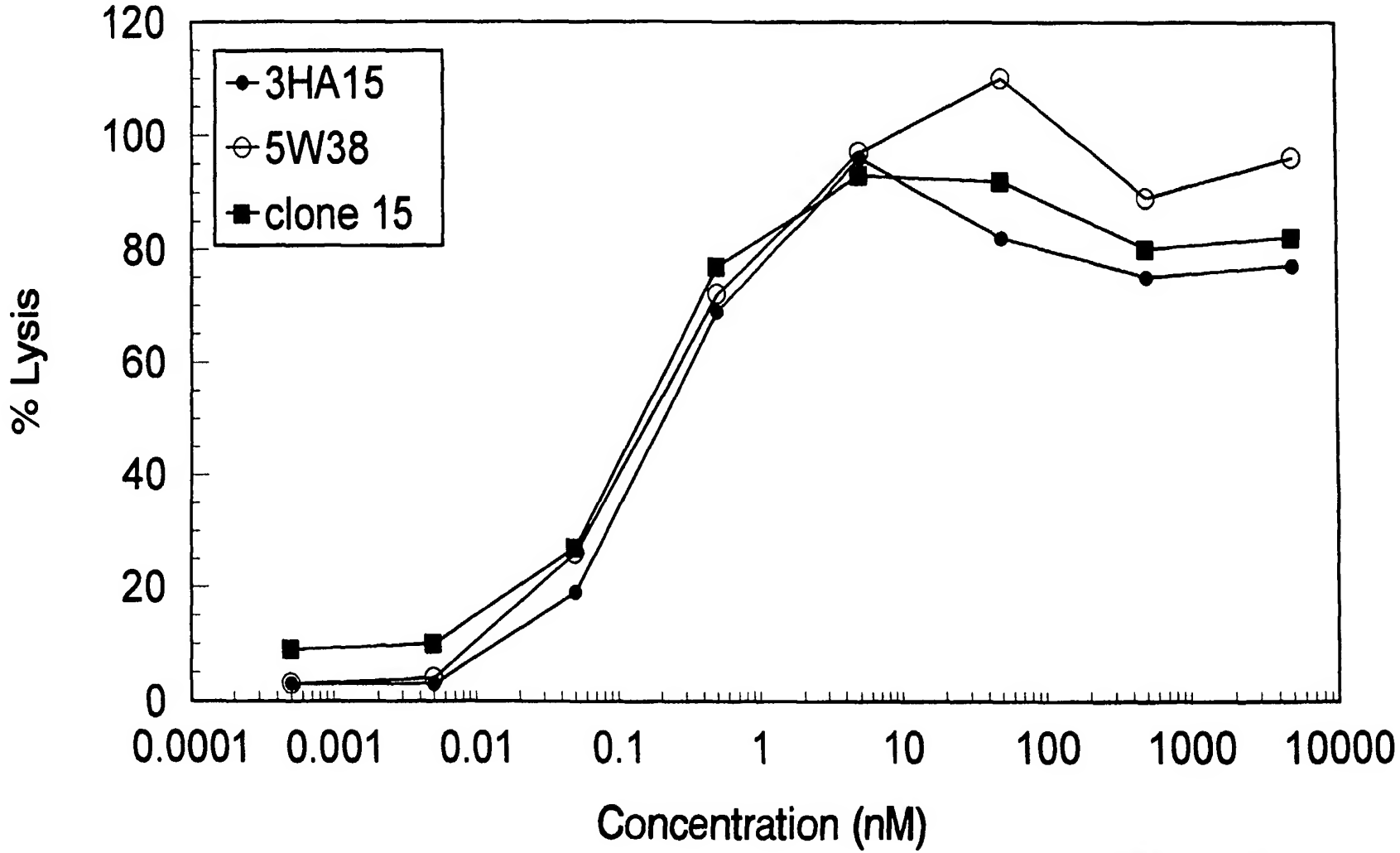

120
100
80
60
40
20
0
% Lysis
3HA15
5W38
clone 15
0.0001
0.001
0.01
0.1
1
10
100
1000
10000
Concentration (nM)

Fig 2b.

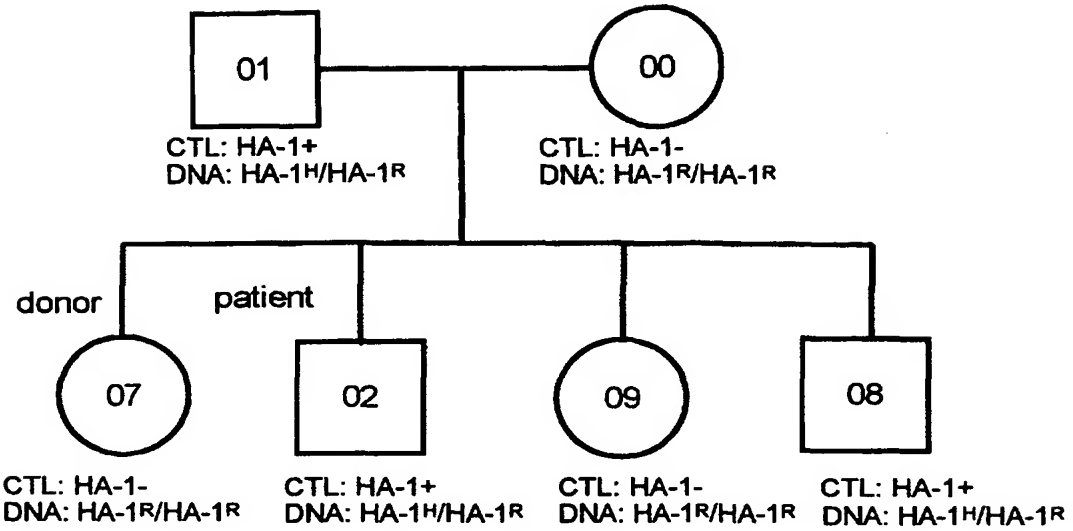
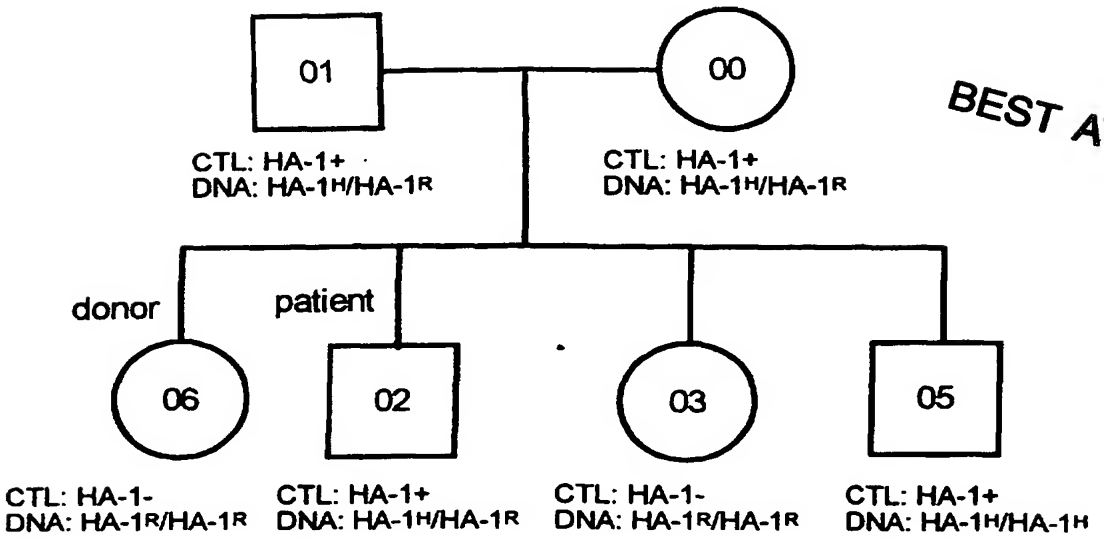

01
00
CTL: HA-1+
DNA: HA-1H/HA-1R
CTL: HA-1-
DNA: HA-1R/HA-1R
donor
patient
07
02
09
08
CTL: HA-1-
DNA: HA-1R/HA-1R
CTL: HA-1+
DNA: HA-1H/HA-1R
CTL: HA-1-
DNA: HA-1R/HA-1R
CTL: HA-1+
DNA: HA-1H/HA-1R

Fig 3A



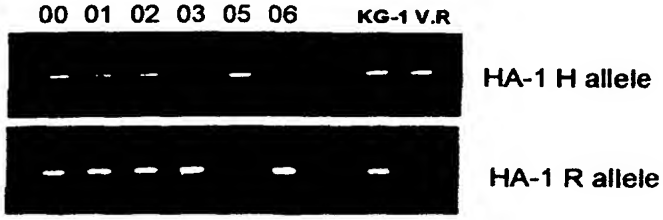


Fig 3b

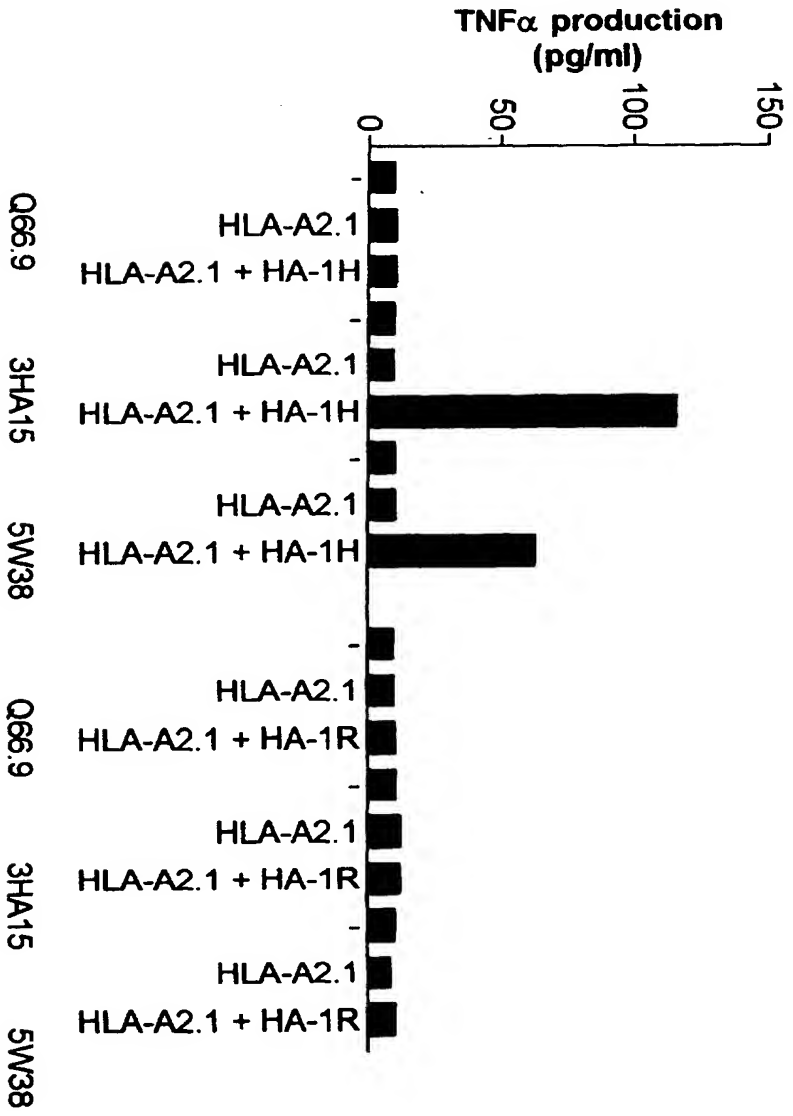


Fig 3C

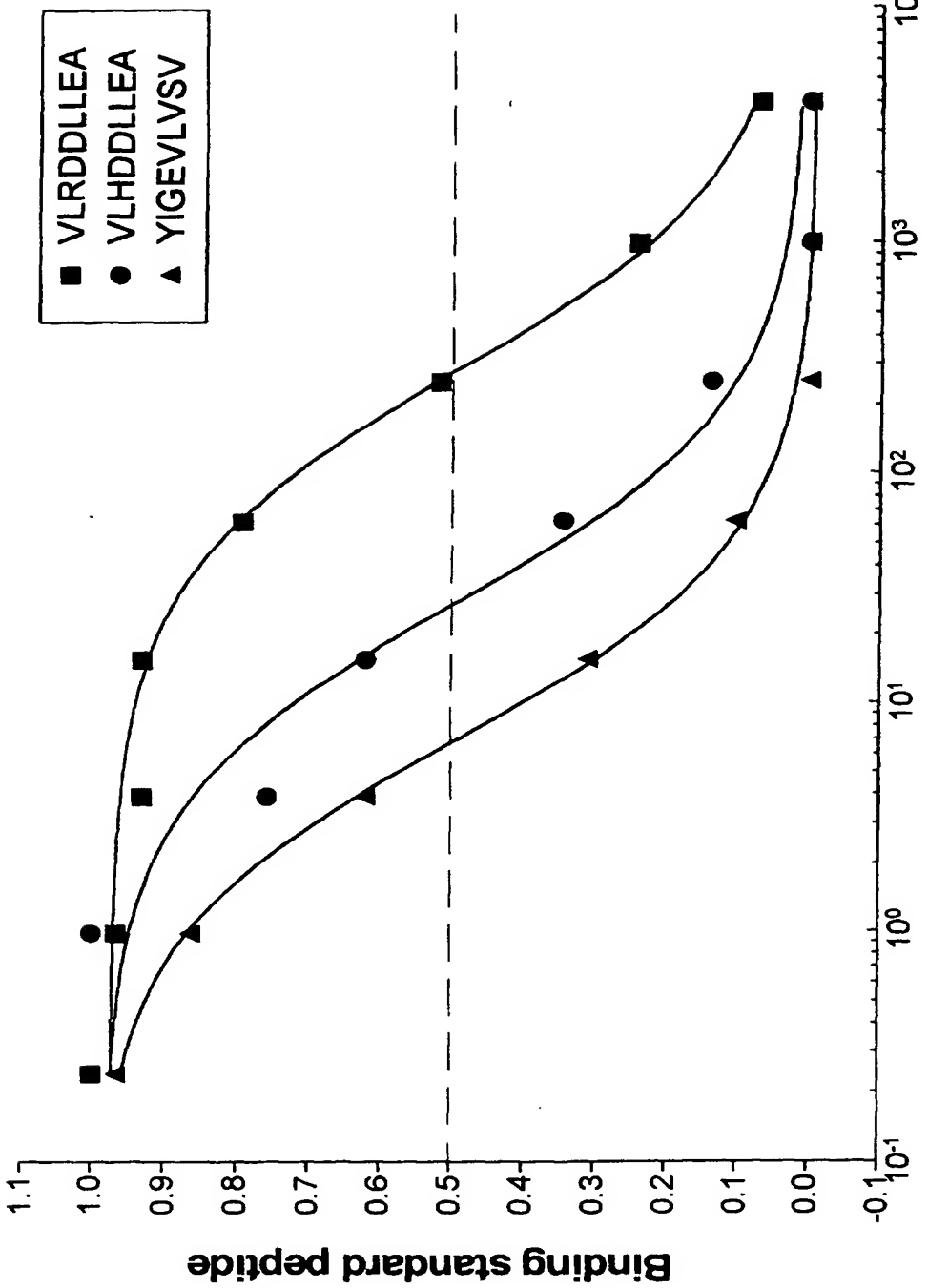

VLRDDLLEA
VLHDDLLEA
YIGEVLVSV
Binding standard peptide
nM peptide
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
10-1
10⁰
10¹
10²
10³
10⁴

Fig 4a

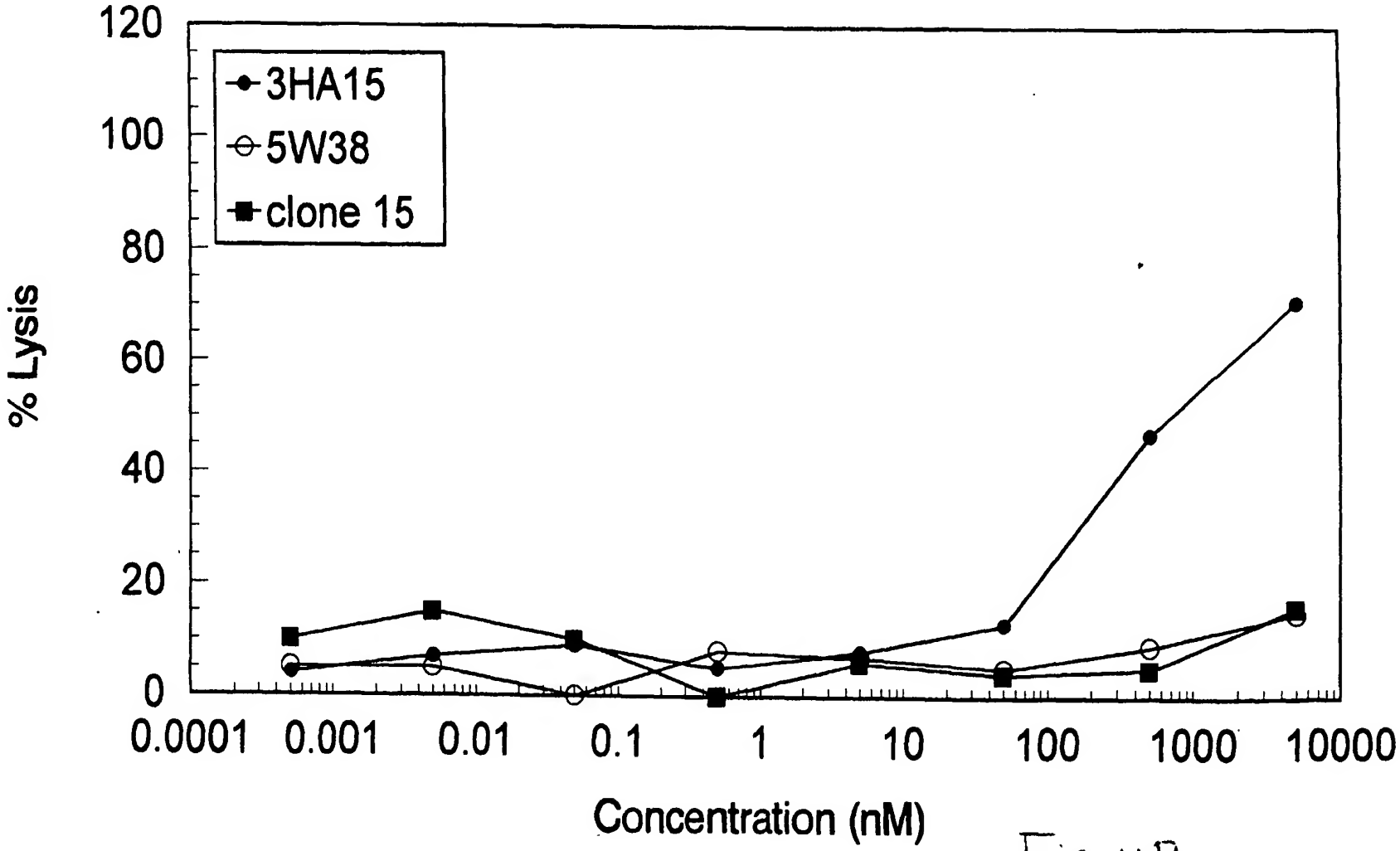

120
100
80
60
40
20
0
% Lysis
3HA15
5W38
clone 15
0.0001
0.001
0.01
0.1
1
10
100
1000
10000
Concentration (nM)

Fig 4B

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

H 20.10.97

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Glu Cys Val Leu His Asp Asp Leu Leu Glu Ala Arg Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Tyr Ile Gly Glu Val Leu Val Ser Val
1 5

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Smulders, Theodorus A.H.J., Ir.
Vereenigde Octrooibureaux
Nieuwe Parklaan 97
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PAYS-BAS

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Datum/Date
12.02.98

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n° /Patent Nr /Patent No./Brevet n°.
Eur 4340	97202303.0 - -

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Rijksuniversiteit te Leiden

COMMUNICATION

The European Patent Office herewith transmits

- ☐ the European search report
- ☐ the declaration under Rule 45 EPC
- ☒ the partial European search report under Rule 45 EPC
- ☐ the supplementary European search report concerning the international application under Article 157(2) EPC relating to the above-mentioned European patent application. Copies of the documents cited in the search report are enclosed.

The following specifications given by the applicant have been approved by the Search Division :

☒ Abstract ☒ Title ☐ Figure

- ☐ The abstract was modified by the Search Division and the definitive text is attached to this communication.
- ☐ The following figure will be published with the abstract, since the Search Division considers that it better characterises the invention than the one indicated by the applicant.

  Figure:

- ☐ Additional copy(copies) of the documents cited in the European search report.

EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS · DEN HAAG · THE HAGUE · LA HAYE

12. 2. 1998

Marcos Bello

REFUND OF THE SEARCH FEE

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 97 20 2303

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	WO 97 05169 A (UNIV LEIDEN ;UNIV VIRGINIA (US); GOULMY ELS A J M (NL); HUNT DONAL) ---		C07K14/705 C07K16/28 A61K38/17 C12N5/06 C12N5/08
A	WO 97 05168 A (UNIV LEIDEN ;GOULMY ELS A J M (NL); HUNT DONALD F (US); ENGELHARD) ---		
A	GOULMY E ET AL: "THE ROLE OF HUMAN MINOR HISTOCOMPATIBILITY ANTIGENS IN GRAFT FAILURE: A MINI-REVIEW" EYE, vol. 9, 1995, pages 180-184, XP000653528 ---		
A	J.M.M. DEN HAAN ET AL: "Conservation of Minor histocompatibility antigens between human and non-human primates" EUR. J. IMMUNOL., vol. 26, 1996, pages 2680-2685, XP002051263 ---		
	-/--		TECHNICAL FIELDS SEARCHED (Int.Cl.6) C07K

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
THE HAGUE	8 January 1998	Cervigni, S

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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EPO FORM 1503 03.82 (P04C07)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 97 20 2303

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A,D	HAAN DEN J M M ET AL: "IDENTIFICATION OF A GRAFT VERSUS HOST DISEASE-ASSOCIATED HUMAN MINOR HISTOCOMPATIBILITY ANTIGEN" SCIENCE, vol. 268, 9 June 1995, pages 1476-1480, XP002020887 -----		TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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EPO FORM 1503 03.82 (P04C10)

Remark : Although claim 8 and 10 are directed - at least partially - to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 97 20 2303

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

08-01-1998

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9705169 A	13-02-97	AU 5409996 A	26-02-97
WO 9705168 A	13-02-97	AU 6631796 A	26-02-97

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COPY

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PAYS-BAS

Datum/Date
09.02.99

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°.
Eur 4340	97202303.0-2106

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Rijksuniversiteit te Leiden

NOTING OF LOSS OF RIGHTS PURSUANT TO RULE 69(1) EPC

The European patent application cited above is deemed to be withdrawn because

(X) no designation of the inventor in due form was filed within the time limit specified in the communication (EPO Form 1045) issued under Rule 42 EPC
(...) no data concerning the inventor was submitted within the time limit specified in the communication (EPO Form 1212) issued under Rule 104b(2) EPC
(...) the correction(s) submitted does (do) not properly remedy the deficiency (deficiencies) indicated in the communication (EPO Form 1045) with regard to the designation of the inventor (Article 91(5) EPC).

POSSIBILITY OF APPEAL:

1. Application for a decision
If the applicant considers that the finding of the European Patent Office is inaccurate, he may, within TWO MONTHS after notification of this communication, apply in writing for a decision on this matter by the European Patent Office (Rule 69(2) EPC). The application can only cause the finding to be set aside if loss of rights has not actually occurred.

2. Application for restitutio in integrum
If the applicant was unable to observe the time limit despite having taken all due care required by the circumstances, he may, upon application, have his rights re-established, provided that the time limits and formal requirements laid down in Article 122 EPC are complied with.

RECEIVING SECTION

T. van der VELDEN
Tel.: 31 70 3403691

REGISTERED LETTER

EINGANGSSTELLE / RECEIVING SECTION / SECTION DE DEPOT

File number : 97202303.0

FINAL INSTRUCTIONS

for closing an application (loss of all rights)

FINDING

1. (X) No use has been made of any of the legal remedies available.
   (X) The decision refusing the: appeal/application for re-establishment of rights/request for further processing has become final.

2. ( ) The application will not be dealt with as a European patent application (Rule 39 EPC).
   (X) The decision refusing the application has become final.
   (X) The application is deemed to be withdrawn.

3. Form 1320 has been despatched (if necessary)

4. The return of Form 1320 is to be controlled by coding "BEEF".

5. Checked with regard to costs; where applicable, refund ordered (RFAC).

INSTRUCTIONS

1. ADWI(3) or REFU(3) and DEAD have been coded.

2. If applicable:
   - Folder has been marked DEAD.
   - Store in "DEAD" archive.

THE HAGUE, ...

RECEIVING SECTION

.....
(Formalities Officer)

27.05.99

T. van der VELDEN
Tel.: 31 70 3403691